# DEVELOPMENT OF AN IN VITRO TECHNIQUE FOR EXAMINING CATCH BOND BEHAVIOR OF INTEGRIN RECEPTORS

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# DEVELOPMENT OF AN IN VITRO TECHNIQUE FOR EXAMINING CATCH BOND BEHAVIOR OF INTEGRIN RECEPTORS

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# INTRODUCTION

Catch bonds are recently discovered receptor-ligand adhesion mechanisms which prolong the lifetimes of bonds between specific molecules as increasing force is applied. Slip bonds, in contrast, have shortened bond lifetimes due to the applied force reducing the energy barrier between the free and bound states. Researchers have shown these catch bonds to be important as bond lifetime regulators between cell surface receptors and their ligands, such as integrin-ligand bonds[1,2]. Integrin receptors can modulate their mechanical coupling to the ligand by grouping together after initial ligand binding. Atomic force microscopy studies have demonstrated single molecule bond lifetimes between integrin $\alpha_5\beta_1$ and the extracellular matrix protein fibronectin increase with applied force[3]. Although catch bond behavior has been characterized on the single molecule level, researchers have yet to understand the effect of groups of catch-bond receptors on binding strength.

The goal of this research is to develop an experimental system capable of analyzing how the catch-bond behavior modulates the binding force of groups of receptors. Our objective is to develop a novel experimental technique that will enable us to examine the catch-bond behavior of groups of integrin receptors in a simplified cell-free system consisting of beads. To achieve the research goal, we will utilize a spinning disc device to measure the binding strength of plastic beads presenting groups of receptors and their surface bound ligands. Specifically, the appropriate ligand will be bound (passively or covalently) to the surface of polystyrene microspheres of similar size to a rounded mammalian cell (Fig. 1-A). Next, the surfaces of glass coverslips will be prepared with model receptors (antibodies) corresponding to the ligand (Fig. 1-B,1-C). The receptor-functionalized beads will then be seeded on to the ligand-functionalized glass surfaces at an optimal density. The spinning disc device will be used to quantify the adhesion strength of the samples.

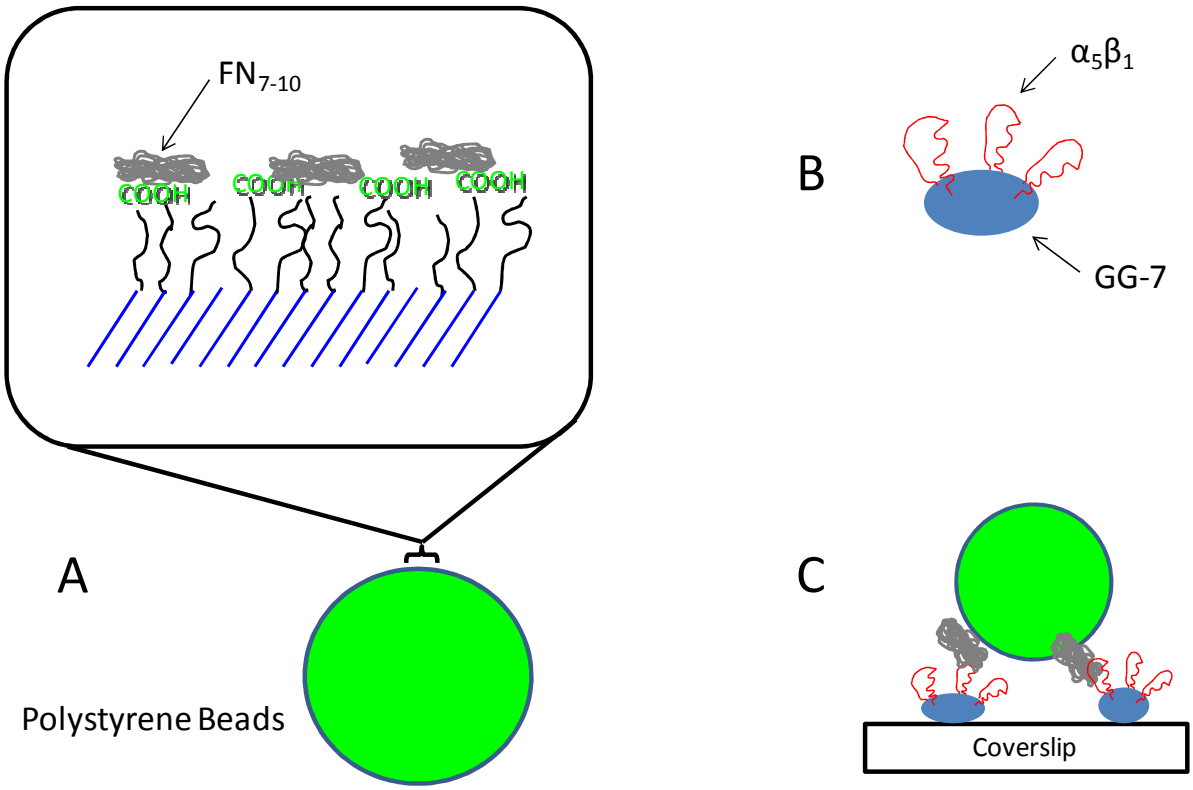


**Figure 1. (A) Expanded view of $FN_{7-10}$ passively adsorbed on carboxylated, polystyrene beads. (B) Fc $\alpha_5\beta_1$ receptor bound to Fc specific GG-7 molecule. (C) Coupled capture antibody and chimera on fibronectin fragment-functionalized glass coverslips.**

Our long-term goal is to use the technique developed in this thesis work to examine the catch-bond behavior of groups of $\alpha_5\beta_1$ integrin molecules. A chimeric $\alpha_5\beta_1$ integrin-Fc molecule, which presents the integrin binding domain at one end of the molecule and the human Fc portion of an antibody at the other end, will be used as the bead-bound receptor while fibronectin and various mutations of the molecule will be used as the surface-bound ligand. This experimental platform will allow the analysis of catch-bond behavior in a well-defined cell-free model system.

## MATERIALS

Fluoresbrite® carboxylate microspheres (10.0μm and 2.0μm particles packaged as 2.5% aqueous suspension) were purchased from Polysciences, Inc. (Warrington, PA) and were used to covalently couple human IgG antibodies onto the beads. These fluorescent monodispersed polystyrene microspheres contain carboxylate groups on their surfaces which can be activated for the covalent coupling of proteins. Dulbecco's phosphate buffered saline (DPBS) was purchased from Invitrogen. Peptide tethering reagents, N-hydroysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-human IgG (Fc specific) clone GG-7 mouse antibody was also acquired from Sigma-Aldrich (St. Louis, MO). Allophycocyanin-AffiniPure goat anti-mouse IgG antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyclonal human IgG was obtained from AbD Serotec (Oxford, United Kingdom). DTSSP (3,3' –dithiobis[sulfosuccinimidylpropionate]) was procured from Thermo Scientific (Rockford, IL). Polystyrene flow cytometry tubes (5mL) were purchased from BD Falcon (Northbrook, IL).

## METHODS

### Conjugation of Capture Antibody to Beads

Conjugation of GG-7 capture antibody to carboxylated, polystyrene beads provides a stable linkage between the antibody and bead surface. The carboxyl groups can either be activated to form esters or covalently joined to the capture antibody by crosslinking. These methods were used to ensure proper immobilization of GG-7 to the polystyrene surface. Since the Fc region of IgG provides a natural specific attachment site for the GG-7 molecules, we proceeded to bind the Fc-specific capture antibody to the Fc region of human IgG. We used reactive aldehydes to react with protein amine groups for the coupling[6].Subsequent incubation and binding of labeled human IgG with the GG-7 conjugated surface would determine if GG-7 was conjugated to the bead surface.

In the event there are not any clear signs of increased GG-7 binding with biotinylated Hu-IgG two techniques would be used in order to optimize GG-7 conjugation: (1) conjugating 1% Bovine Serum Albumin (BSA) through NHS/EDC chemistry then covalently linking GG-7 by

DTSSP or (2) conjugating 1% Bovine Serum Albumin (BSA) through NHS/EDC chemistry then covalently linking GG-7 by NHS/EDC chemistry again.

In this study, Wash/Storage and Coupling Buffers were prepared prior to bead preparation. For tethering of antibody, all microspheres were initially rinsed with Coupling Buffer and activated for 15 minutes with 20μL of NHS/EDC solution (3:1 ratio of EDC to NHS). At a concentration of 10μg/mL, the GG-7 capture antibody was expose to the beads for 30 minutes to covalently tether. A 1% dilution of BSA was then added to each mixture to prohibit any non-specific binding of antibodies on the FITC-labeled bead surface. When using the DTSSP cross-linker, the amine-reactive sulfo-NHS ester at each end of the spacer arm would react swiftly with the amine-rich GG-7 molecule[7]. On the other hand, using sulfo-NHS/EDC hydrolyzes more slowly in water than DTSSP. The combination of sulfo-NHS and EDC increases the half-life of the reaction and the conjugation yield of GG-7[8]. Although the reaction between DTSSP and the capture antibody reacts at a faster rate than through NHS/EDC chemistry, the NHS reaction decays more slowly allowing GG-7 additional time to form additional bonds. Therefore, conjugation of 1% BSA through NHS chemistry would be expected to produce a higher level of bound GG-7 on the bead surface.

**Engineered Fc Chimera**

Previously developed by our collaborators, a chimeric $\alpha_5\beta_1$ has been engineered to express the human Fc antibody domain. Expression of the Fc tag provides a molecular "handle" for easier purification of presentation[4]. The carboxylated, polystyrene beads must be functionalized in order to optimize binding of the Fc-chimera to its surface. The focus is to determine if conjugating an Fc specific capture antibody, GG-7, to the FITC-labeled bead surfaces or passively adsorbing human IgG proteins in order to optimize and maintain bead functionality. We captured purified chimeric $\alpha_5\beta_1$ integrin-human Fc protein to the bead surface by incubation of the functionalized beads with the purified chimeric protein. These steps produced chimeric $\alpha_5\beta_1$ integrin receptor presenting polystyrene beads for studying how catch bond behavior modulates the binding force of groups of receptors.

**Coupling GG-7 and $\alpha_5\beta_1$ to $FN_{7-10}$-Coated Beads**

Coupling and Wash/Storage buffers were prepared before the experiment. Given that GG-7 is raised in a mouse, Gt-$\alpha$Ms APC was used to fluorescently tag the GG-7 for flow studies to quantify density of immobilized GG-7. Tagged GG-7 solutions were also prepared prior to the experiment so binding of capture antibody to chimera and bead preparation could be run simultaneously. In bead preparation, we had to ensure there were at least 80,000 beads per condition; therefore for 6 conditions we used a total volume of 11 $\mu$L of bead solution. $FN_{7\text{-}10}$ (10 $\mu g/mL$), a recombinant fragment which spans from the seventh to tenth domain of human fibronectin and represents the ligand for the integrin receptor, was passively adsorbed to the carboxylated bead surface for 30 minutes[9]. The beads were then "blocked" with BSA to prevent any non-specific binding on the surface. Two buffers were used as controls for the proper activation of the chimera on the bead surface, 2mM $Mn^{2+}$ $PBS^{+/+}$ and 10mM EDTA in $PBS^{-/-}$ [10]. Each condition was incubated with its respective combination of chimera and antibody for 30 minutes to ensure specific binding to $FN_{7\text{-}10}$. Solutions were placed into 5 mL flow cytometry tubes for flow studies.

***Spinning Disc Assay***

The adhesion strength of model cells was measured using a spinning disc assay previously developed by García et al[4]. This device draws fluid from its surroundings to apply a range of shear forces to model cells attached to the disc surface. The discs containing adherent model cells are mounted on a platform and spun at a constant speed in flow chamber that approximates an infinite fluid. This causes the fluid to approach the disc in its center and exit radially. A detachment force is thereby produced which is governed by the following equation:

$$\tau = 0.8r\sqrt{\rho\mu\omega^3}$$

where $\tau$ is the resultant shear stress, r is the radial distance from the center of the disc, $\rho$ is the fluid density, $\mu$ is the fluid viscosity, and $\omega$ is the rotational speed of the disc. In this assay, a linear range of detachment forces is applied to the disc surface where this shear force increases from the center to the edge of the disc. By counting the number of beads at different radial positions after spinning, a detachment profile (bead number vs. applied shear stress) can be generated and the shear stress for 50% detachment, which represents the mean adhesion strength, can be calculated.

## RESULTS

### *Conjugation and Adsorption of GG-7*

Previous studies have shown that GG-7 capture antibody binds specifically to human Fc domain and every Hu-IgG antibody has the Fc domain. The process of attaching the capture antibody to the bead surface had yet to be determined. Using 1% BSA coated beads as a control for specificity, the capture antibody was either conjugated via peptide chemistry or passively adsorbed to the polystyrene beads to determine which presentation would preserve its ability to bind Hu IgG molecules. An anti-mouse fluorescent antibody, Gt-αMs APC, was used as a probe for the GG-7 molecule in order to quantify GG-7 molecules on the FITC-labeled bead surfaces through flow cytometry. If the GG-7 molecules were active it should bind to Hu-IgG molecules.

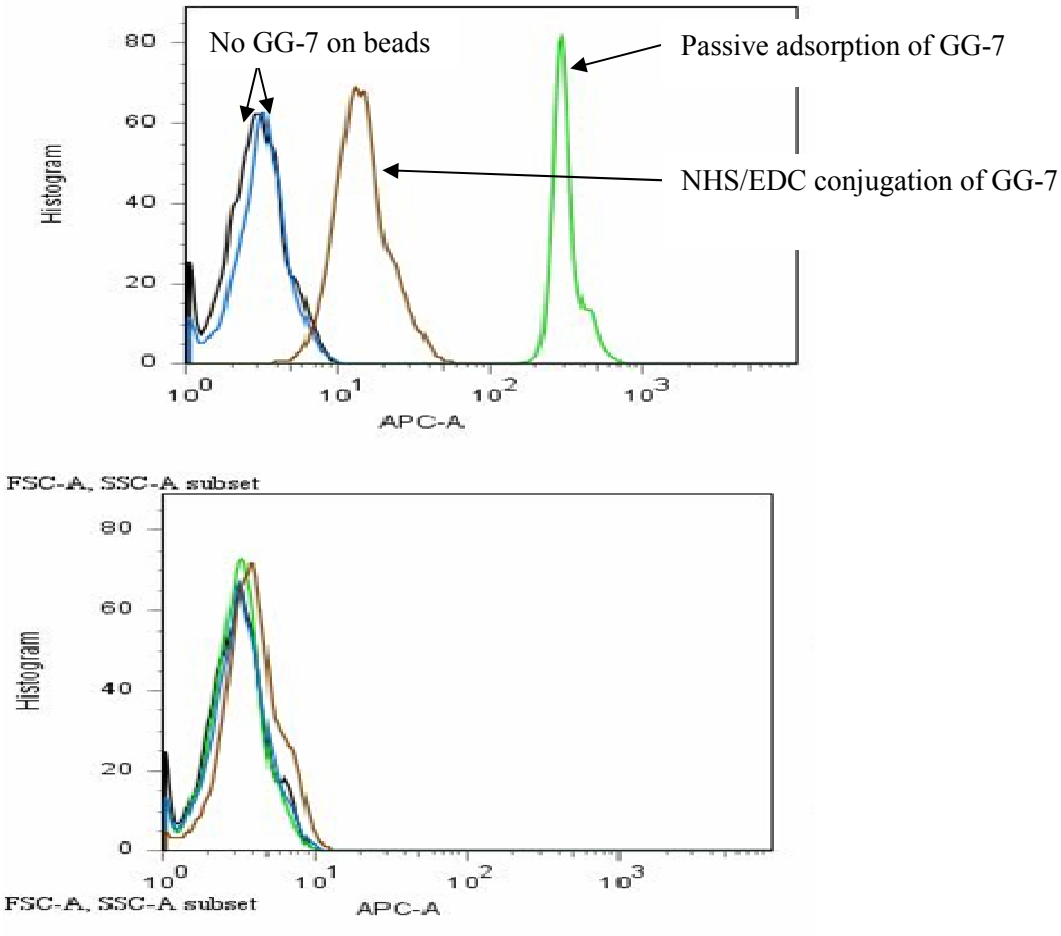


**Figure 1: (Top) Comparison of passive adsorption and conjugation of GG-7 capture antibody on FITC-labeled bead surface through NHS/EDC chemistry. The black line represents 1% BSA-coated beads with no**

**GG-7 and the blue line represents conjugated bead surfaces with no GG-7. (Bottom) APC count histogram for examining non-specific binding.**

Figure 1 (top) shows that the APC count of passively adsorbed GG-7 is higher than that of the negative controls, BSA only (black) and anti-rabbit IgG non-specific control (blue). Although conjugating GG-7 to the polystyrene bead surface through NHS/EDC chemistry increases the APC count from the negative controls, the amount of Gt-αMs APC bound to the surface was still lower than the passively adsorbed case. Therefore, passive absorption of GG-7 to the carboxylated polystyrene beads appears to retain the functionality of the capture antibody. The bottom panel in Figure 1 shows the bead APC count with a non-specific anti-rabbit antibody. The low APC count ensures that there is no non-specific binding given GG-7 capture antibody is specific to anti-mouse antibodies rather than anti-rabbit.

***Passive Adsorption of Human IgG***

The results of conjugating GG-7 to the FITC-labeled bead surface and binding human IgG were not favorable in terms of binding human IgG, therefore we reversed the procedure. Human IgG was passively adsorbed to the polystyrene beads (Fig. 2-A). The specific location of GG-7 attachment to human IgG was characterized as the Fc region of the IgG molecule (Fig. 2-B). Fluorescently tagged GG-7 was then bound to the human IgG-coated bead surface (Fig. 2-C). This would determine if GG-7 could recognize the human IgG antigen when GG-7 was in solution and not conjugated or passively adsorbed to the bead surface.

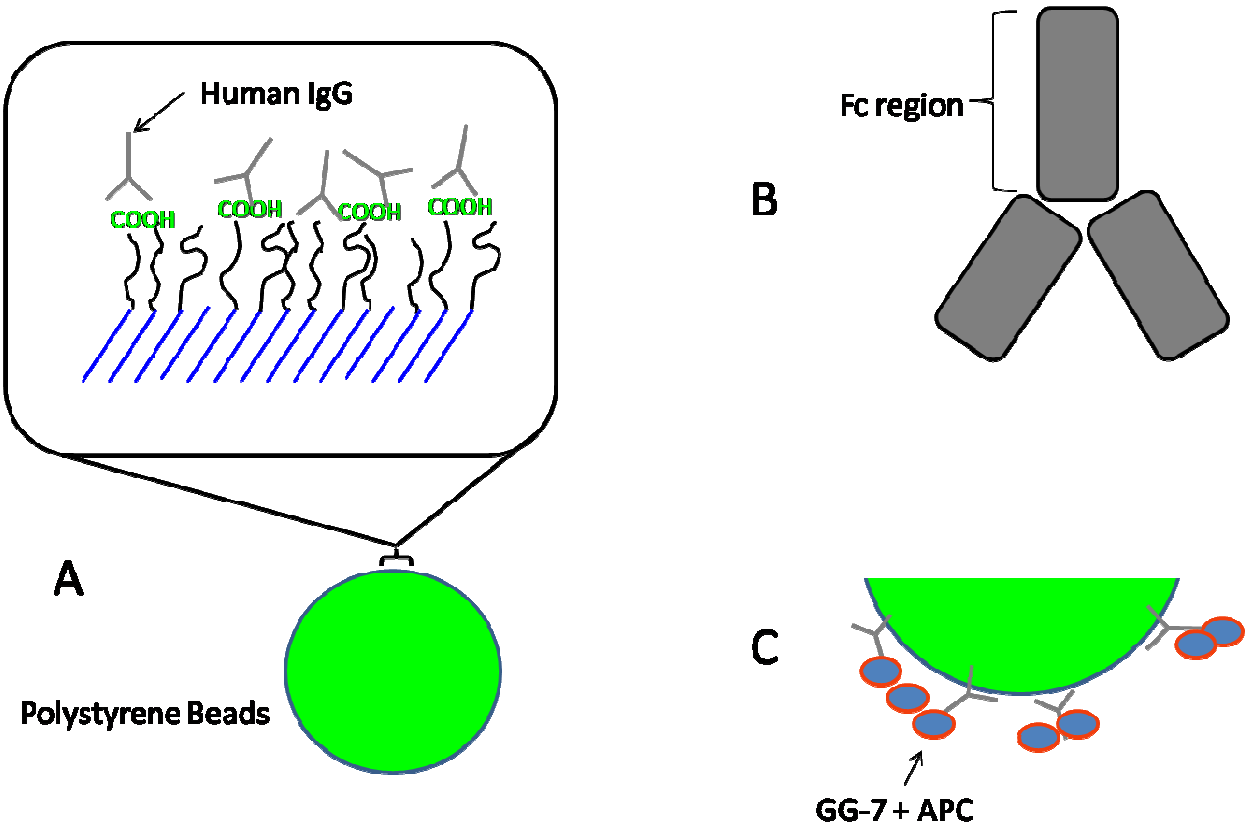


**Figure 2. (A) Diagram of passively adsorbed human IgG on bead surface. (B) Human IgG antigen highlighting the fragment crystallizable region at its tail. (C) Fluorescently tagged GG-7 molecules binding specifically to the Fc region of human IgG.**

**Table 1: Controls for passive adsorption of human IgG**

| | Solutions | | Bead Coatings |
|---|---|---|---|
| A | GG-7 + Gt-αMs APC | 1 | Hu-IgG |
| B | $PBS^{+/+}$ + Gt-αMs APC | 2 | Rb-IgG |
| | | 3 | No IgG |

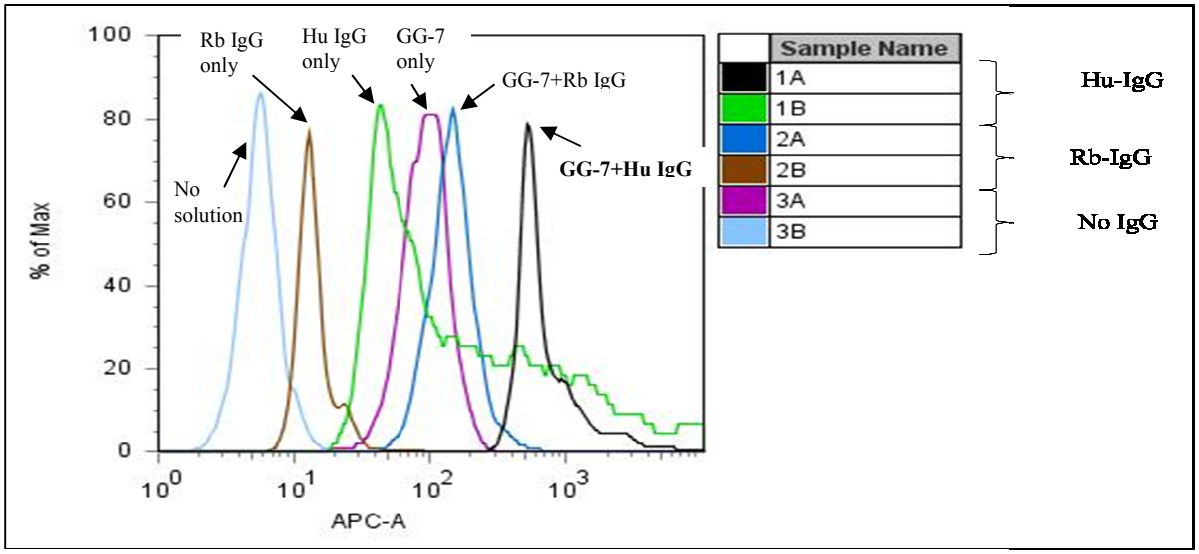


**Figure 3: GG-7 bound to human IgG functionalized beads using flow cytometry.**

Table 1 displays the controls used in the passive absorption of human IgG and the corresponding flow cytometry results are displayed in Figure 3. GG-7 specifically bound passively adsorbed IgG on the bead surface. There was significantly less binding of GG-7 to beads coated with Rb-IgG than human IgG where the binding level of GG-7 to Rb IgG was nearly the same as the bead coated with BSA.

We were able to successfully passively adsorb human IgG (antigen) to 10 µm FITC-labeled polystyrene beads which we then incubated with GG-7 capture antibody tagged with Gt-αMs APC. Next, we set out to determine whether $\alpha_5\beta_1$ Fc chimera can bind fibronectin fragment $FN_{7\text{-}10}$ in our system. Therefore, we adsorbed $FN_{7\text{-}10}$ to the bead surface and incubated the glass coverslip surface with a combination of $\alpha_5\beta_1$ and GG-7. GG-7 should specifically bind to $\alpha_5\beta_1$ through the Fc domain. Table 2 shows the buffers and bead conditions used for this study. Figure 4 presents the results of incubating GG-7 and chimera to the $FN_{7\text{-}10}$-coated bead surface.

**Table 2: Conditions for binding Fc chimera to $FN_{7-10}$.**

| Beads | | Buffers | | Solutions | |
|---|---|---|---|---|---|
| A | $FN_{7-10}$ | 1 | $PBS^{+/+}$ | a | GG-7 + $\alpha_5\beta_1$ + Gt-αMs APC |
| B | BSA | 2 | 2mM $Mn^{2+}$ $PBS^{+/+}$ | b | $\alpha_5\beta_1$ + Gt-αMs APC |
| | | 3 | 10mM EDTA in $PBS^{-/-}$ | c | Gt-αMs APC |

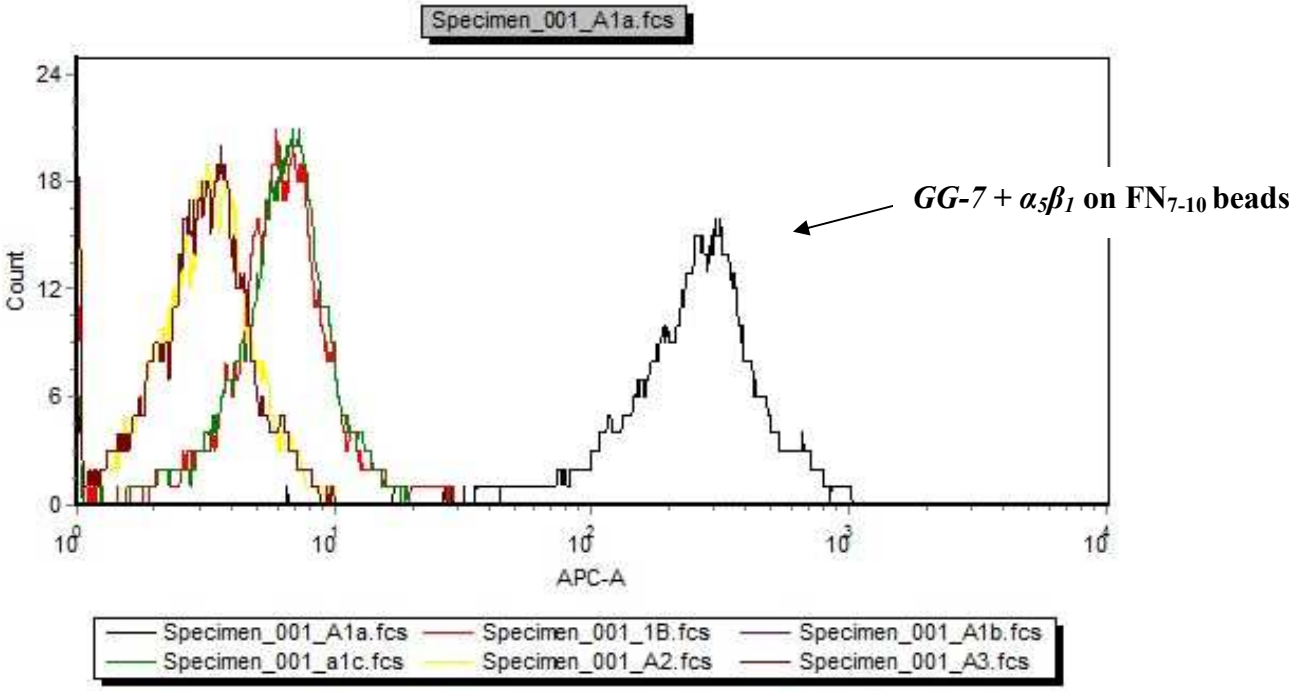


**Figure 3: $FN_{7-10}$ binding to on polystyrene bead surface.**

We were detected beads with bound GG-7 with Gt-αMs APC following flow studies. As the bottom panel in Figure 3 illustrates, condition A1a ($FN_{7-10}$ bound to fluorescently tagged GG-7 and $\alpha_5\beta_1$ in $PBS^{+/+}$ buffer) had a significantly high binding specificity between the fibronectin fragment-functionalized beads and the solution consisting of Fc-specific GG-7 and integrin-Fc chimera. Incubating integrin-Fc chimera alone to the $FN_{7-10}$ bead surface had higher binding than with the BSA coated surface; however, the binding was considerably lower than incubation with GG-7 and the chimera. These results indicate beads coated with $FN_{7-10}$ can effectively bind Fc-chimera in our cell-free system.

***Varying Bead Density and Seeding Volume***

For the spinning disc assay, the FITC-labeled bead density must be high enough to yield results with high statistical reliability but low enough as to avoid bead-bead interactions during fluid flow. The amount of beads per disc was varied to determine which produced a disc with enough beads for proper analysis. Several bead counts were studied (k = 1,000): 800k, 500k, and 250k. The 250k and 500k discs did not present beads which were evenly dispersed across the glass disc surface. The 800k condition presented discs with a dense allotment of beads on the disc surface; however, the overwhelming density caused the beads to clump together in some areas. The bead count was therefore reduced by 15% making the optimal bead count 680k per disc. To seed the coated beads onto the glass discs, the bead solution was applied directly to the surface. The seeding volume for this experiment was 450μL per disc as other seeding volumes were also tested to maximize bead uniformity.

Beads dispensed in the center of the discs tended to assemble in the same area, while the amount of model cells on the outer edges of the 25mm discs have been observed to be sparse (Figure 3 Top). When these model cells collide during seeding, the affinity between each model cell increases causing the beads to congregate at the point of seeding. A novel seeding technique used to eliminate the central-dominant bead seeding involved the spiral dispersion of beads on the disc surface. Commencing at the center of the disc, the beads were dispensed in three, continuous spiral motions towards the edges of the disc[8]. This spiraling technique produced discs with a more uniform seeding pattern than direct application to the center (Fig 3 Bottom).

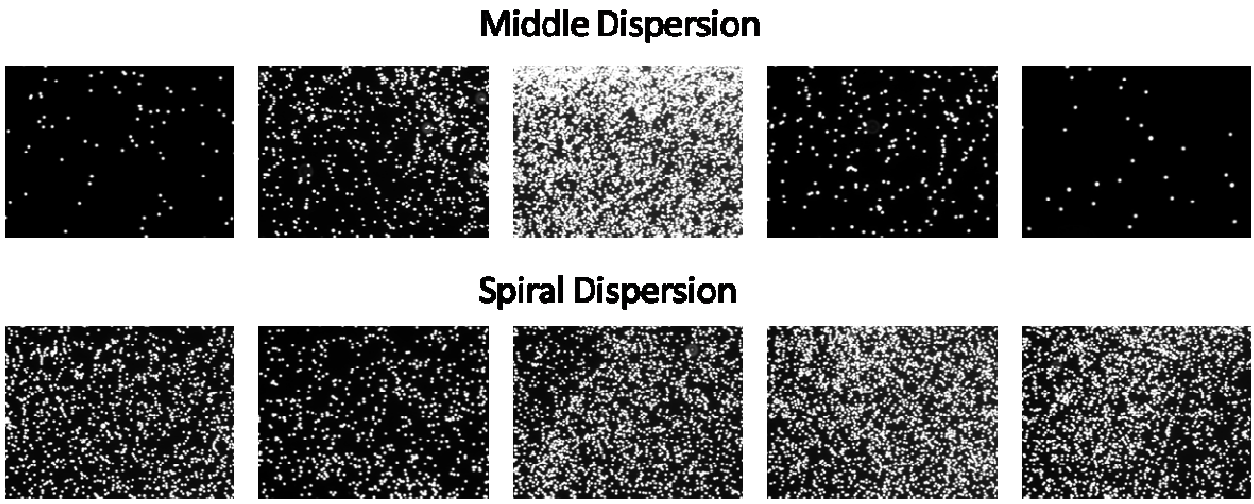


**Figure 3. (Top) Center-dominant bead seeding from direct dispersion of bead solution to middle of glass coverslips. (Bottom) Uniform bead seeding using spiral technique.**

### *Maximizing Adhesion of Beads on Surfaces*

Pilot studies revealed poor stable adhesion of the antibodies on the glass discs for spinning experiments. To overcome this limitation, we introduced HDT, a self-assembled monolayer (SAM), to the disc surface to increase the adsorption strength between the beads and the discs. A rabbit antibody against human fibronectin (RbαFN) was incubated onto the surface of HDT coated on Au/Ti deposited glass discs for 30 min and $FN_{7\text{-}10}$ was passively adsorbed to 10μm carboxylated beads. We used the spinning disc assay to measure the adhesion strength between the ligands and its antibody. At low speeds, ~200 rpm, the shear force applied to the surface would displace a significant amount of beads off the surface. Figure 4 illustrates a comparison of binding RbαFN to $FN_{7\text{-}10}$ –coated beads with and without HDT SAMs.

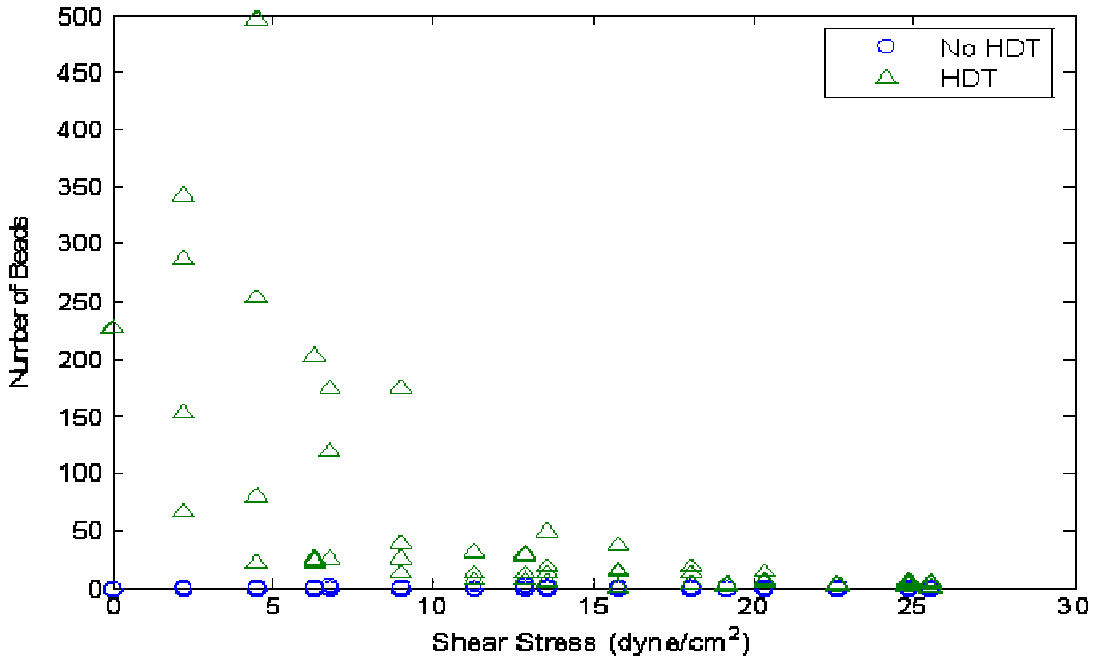


**Figure 4. Comparison of HDT-coated discs to discs without HDT at 200 rpm.**

Figure 4 shows that the addition of HDT to the disc surface increased to the adhesion force between the bead and the disc at 200 rpm. This self-assembled monolayer was able to passively adsorb RbαFN and maintain its ability to bind the $FN_{7\text{-}10}$. The detachment profile was observed to be sigmoidal for the HDT condition as expected[4]. The detachment force on the disc was applied from the center of the disc and moved outwards causing the perimeter beads to be displaced. Although the application HDT was successful in increasing the amount of adherent beads on the surface, the speed of 200 rpm is relatively low for our studies. Smaller microspheres should reduce the amount of shear stress on the beads while reducing its area on the surface as described in the following equation[11]:

$$F_d = \frac{1}{2} C_d \rho v^2 A$$

where $F_d$ is the drag force on the bead, $C_d$ is the dimensionless drag coefficient, $\rho$ is the fluid density, $v$ is the speed of the object relative to the fluid, and A is the cross-sectional area of the bead. The drag force equation shows with a reduction in the cross-sectional area of the bead and holding all other parameters constant, the resulting drag force on the microsphere also decreases. This provides the opportunity to increase the speed of the spinning disc device without a significant loss of beads. Therefore, we used 2μm beads to be able to study how smaller beads

would affect their displacement. In this case, 1% BSA-heat denatured beads were adsorbed to the RbαFN disc surface with HDT. The 2μm polystyrene beads were spun at 200 rpm and no spinning (0 rpm).

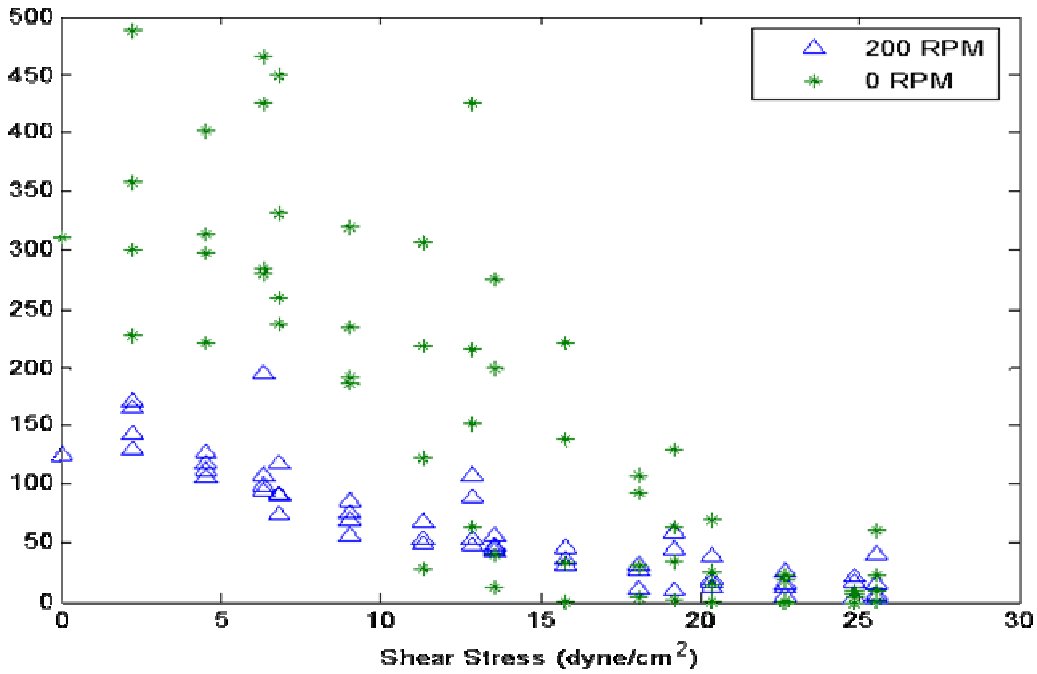


**Figure 5. Sigmoidal curve of detached 2μm polystyrene beads.**

As shown in Figure 5, the detachment of model cells was sigmoidal where the number of bound beads decreased with the increase in spinning speed from no spinning to 200 rpm. This data indicates the possibility of characterizing the effects of shear stress on groups of receptors. We would be able to determine how the structure of the integrin receptor changed due to the applied stress of the spinning disc.

## DISCUSSION

The central goal of this study was to develop an experimental system to characterize catch bond behavior in $\alpha_5\beta_1$-Fc chimeric integrin. One major obstacle was determining the orientation of the antigen and antibody on a polystyrene bead surface. We needed to determine whether it was more feasible to conjugate GG-7 capture antibody (which binds human IgG) or passively adsorb human IgG onto a FITC-labeled polystyrene bead surface and maintain bead functionality. A preceding study in this project revealed antibodies could successfully be

conjugated to the carboxylated bead surface. Therefore, conjugating GG-7 through NHS/EDC chemistry inhibited the functionality of the GG-7; the conjugation significantly reduced to amount of biotinylated human IgG bound on the surface relative to the passively adsorbed case. Results showed that neither covalently or passively adsorbed GG-7 to FITC-labeled beads was able of binding human IgG. We reverse the coupling strategy so that $FN_{7\text{-}10}$ was bound to beads and GG-7 was passively adsorbed to the disc surface. In this case, GG-7 was able to specifically recognize human IgG. Passively adsorbing $FN_{7\text{-}10}$ to surface maintained bead functionality and, with the addition of Fc chimera, Fc-specific GG-7 antibody was successfully bound to the bead surface through the Fc domain of the chimera. These differences probably arise from differences in adsorption of biomolecules between carboxylated polystyrene and glass.

The bead density was varied to prevent clumping of beads on the glass discs. Bead clumping would reduce the accuracy of data when using the spinning disc assay as it requires less force to dislodge a cluster of beads with a higher surface area than a single microsphere. We also observed that the polystyrene beads showed center-dominated adhesion when dispensing the bead solution in the middle of the discs. This phenomenon occurred because of the affinity between the polystyrene beads joining at the point of application. A tested spiral technique was used to sustain an even dispersion of model cells. To further optimize our experimental system, we introduced a self-assembled monolayer (HDT) to Au-plated disc surfaces where the head group of the HDT has a special affinity for Au. The addition of HDT increased the amount of receptor functionalized beads on the Au-plated disc surface. This added adhesive strength was only aided when the model cell size was reduced to increase the bead's resistance to the applied shear stress of the spinning disc assay. The results of this study indicate the possibility of successfully characterizing the catch-bond behavior of $\alpha_5\beta_1$Fc chimera.

## CONCLUSION

Studies were conducted to understand the effects of binding Fc-specific GG-7 to an Fc human IgG antigen on carboxylated, FITC-labeled beads. The results indicate conjugated GG-7 can bind human IgG antigen; however, significantly diminishing its functionality. Contrarily, GG-7 was able to specifically recognize passively adsorbed human IgG. Passive adsorption of $FN_{7\text{-}10}$ fragment incubated with bound $\alpha_5\beta_1$-Fc receptor to the Fc-specific capture antibody successfully maintained the functionality of the polystyrene beads. Experiments were conducted

to determine how the adhesion strength between bonds changed with an applied shear stress from a spinning disc microscopy assay. Quantification of spinning results indicate that we can examine how the molecular structure of the chimera changes with applied shear stress using spinning disc microscopy.

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